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Chromosomal Stabilization of the Proteinase Genes in *Lactococcus lactis*

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The plasmid-encoded proteinase genes *prtP* and *prtM* of *Lactococcus lactis* subsp. *cremoris* Wg2 were integrated by a Campbell-like mechanism into the *L. lactis* subsp. *lactis* MG1363 chromosome by using the insertion vector pKLG610. Two transformants were obtained that differed in the number of amplified pKLG610 copies in head-to-tail arrangements on their chromosomes; MG610 contained approximately two copies, and MG611 contained about eight copies. The amplifications were stably maintained during growth in milk in the absence of antibiotics. The proteolytic activity of strain MG611 was approximately 11-fold higher than that of strain MG610 and about 1.5 times higher than that of strain MG1363(pGKV552), which carried the proteinase genes on an autonomously replicating plasmid with a copy number of approximately 5. All three strains showed rapid growth in milk with concomitant rapid production of acid. The results suggest that a limited number of copies of the proteinase genes *prtP* and *prtM* per genome is sufficient for good growth in milk.

Lactococcus lactis is one of the lactic acid bacterial species of major economic importance in dairy fermentations. The main function of lactococci in milk fermentations for the production of cheese is the rapid production of lactic acid from lactose. Nowadays, the large-scale fermentations in the dairy industry require the availability of genetically stable strains. Two unstable plasmid-encoded properties are of vital importance for good growth and concomitant rapid production of lactic acid: the ability to ferment lactose, a process which involves several enzymes, and the ability to degrade caseins by a cell envelope-associated proteinase (for reviews, see references 6 and 22). The initial degradation of caseins by proteinase and subsequent hydrolysis of the breakdown products by a variety of peptidases enable *L. lactis* to obtain sufficient amounts of essential amino acids, which are present in milk in concentrations too low to sustain good growth (30).

Two types of proteinases can be distinguished with respect to their specificity toward caseins. The PIII-type proteinase degrades both α- and β-casein, whereas the PI-type proteinase only hydrolyzes β-casein, with a specificity different from that of the PIII-type proteinase (38, 39). The amount and type of proteinase produced are also important for the organoleptic properties of the fermentation product (24, 25, 29, 40).

A number of plasmid-located proteinase genes of *L. lactis* encoding PI- or PIII-type proteinases have been cloned and sequenced (7, 17, 19, 21, 41). Detailed analyses revealed that two genes, designated *prtP* and *prtM*, are necessary for proteolytic activity. The *prtP* gene encodes a cell envelope-associated serine proteinase of approximately 200 kDa that is activated by a PrtM-dependent maturation step (for a recent review, see reference 18).

The requirement of genetically stable *L. lactis* strains implicates the stabilization of the genes essential for the dairy industry, such as the proteinase genes. Integration of these genes into the *L. lactis* chromosome may offer a useful approach to achieving this goal. In previous work we described the integration of plasmids that were unable to replicate in *L. lactis* via homologous recombination (26, 27). Stable single-copy and tandemly arranged multicopy integrations were obtained in *L. lactis* MG1363 (27). Similar results were reported by Chopin et al. for strain IL1403 (5).

In this report we describe the integration into the *L. lactis* subsp. *lactis* MG1363 chromosome of the *L. lactis* subsp. *cremoris* Wg2 proteinase genes by a single homologous recombination event (Campbell-like integration) with a pTB19-based vector. Transformants were obtained that carried different numbers of stable tandemly integrated plasmid copies. The effect of the number of integrated proteinase gene copies in the transformants on the growth rate and acid production in milk is discussed.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The strains and plasmids used in this study are shown in Table 1. *Bacillus subtilis* was grown on TY broth and agar plates (35). *L. lactis* was cultured in M17 broth containing 0.5% glucose (GM17; 37) or in 10% (wt/vol) reconstituted skim milk (Oxoid Ltd., London, England) containing 0.5% (wt/vol) glucose. For proteinase isolations, cells were grown on 5% whey permeate containing 0.1% Casitone, 0.5% glucose, and 2% β-glycerophosphate (9). To distinguish proteinase-producing (Prt^+) cells from proteinase-deficient (Prt^-) variants, the lactococci were grown on agar-based reconstituted skim milk plates containing 0.5% (wt/vol) glucose and 0.005% (wt/vol) bromocresol purple (GMAB plates) (21). Recombinant bacterial strains were grown in the presence of the appropriate antibiotics, unless stated otherwise. Erythromycin was used at a concentration of 5 μg/ml for both *B. subtilis* and *L. lactis*. Kanamycin was used at a concentration of 100 μg/ml for *B. subtilis* protoplasts during regeneration on DM3 plates (3) and at a concentration of 5 μg/ml for subsequent growth.

DNA isolation and manipulation. Plasmid DNA was isolated from *B. subtilis* as described by Birnboim and Doly (2) or by the method of Ish-Horowicz and Burke (12). Minipreparations of plasmid DNA from *L. lactis* were prepared as described before (26). *L. lactis* chromosomal DNA was isolated by using a mini-isolation procedure described previously (27). The restriction enzymes, Klenow enzyme, and

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant properties	Source or reference
Bacteria		
B. subtilis PSL1		33
L. lactis		
MG1363	Em^s Prt^-, plasmid free	9
MG1363(pGKV552)	Em^r Prt^+, MG1363 carrying plasmid pGKV552	Laboratory collection
MG610	Em^r Prt^+, MG1363 carrying two to three copies of pKLG610 in the chromosome	This work
MG611	Em^r Prt^+, MG1363 carrying eight to nine copies of pKLG610 in the chromosome	This work
Plasmids		
pKM1	pUC7 carrying a 1.4-kb fragment from pJH1 in the *Pst*I site; this fragment carries a Km^r gene (38)	16
pGKV552	Em^r Prt^+, a pWVO1-derived vector containing the complete *prtP* gene and a functional *prtM* gene, based on pGKV550 (11)	Laboratory collection
pGKV562	Em^r Prt^+, pGKV552 with an additional *Bgl*II restriction site	This work
pGKV562K	Em^r Km^r Prt^-, pGKV562 with the Km^r gene inserted into the *Bam*HI restriction site	This work
pKL400B	Em^r, based on pTB19 and pBR322, carrying 1.3-kb chromosomal insert B	27
pKL401B	Em^r, pKL400B from which the pBR322 origin of replication has been deleted	This work
pKLG600	Em^r Km^r Prt^-, pKL401B carrying the *prtP*, *prtM*, and the Km^r gene of pGKV562K	This work
pKLG610	Em^r Prt^+, pKLG600 from which the Km^r gene has been deleted	This work

T4 DNA ligase were obtained from Boehringer GmbH, Mannheim, Germany, and were used according to the instructions of the supplier. General molecular cloning techniques were carried out essentially as described by Maniatis et al. (28).

Construction of the integration plasmid. The pTB19-pBR322-based integration vector pKL400B, carrying the 1.3-kb lactococcal chromosomal fragment B, has been described before (27). The origin of replication of pBR322 was removed by *Pvu*II digestion and subsequent religation, resulting in plasmid pKL401B.

Plasmid pGKV552 is a derivative of pGKV500 and pGKV550 (11, 21) and contains the complete *prtP* gene and a functional *prtM* gene from *L. lactis* subsp. *cremoris* Wg2. A second *Bgl*II restriction site was introduced in pGKV552 (the first being located downstream of the *prtP* gene) as follows: after digestion of the unique *Xba*I restriction site downstream of *prtM*, the recessed ends were filled in with the Klenow enzyme and then the vector was ligated to a synthetic 10-mer (5′-AAAGATCTTT-3′) *Bgl*II linker, resulting in pGKV562. To facilitate the easy transfer of the proteinase genes to other plasmids, the unique *Bam*HI restriction site within the coding sequence of the *prtP* gene was used to insert an 1.4-kb *Bam*HI fragment of pKM1 (16) carrying the kanamycin resistance (Km^r) gene of pJH1 (38), resulting in pGKV562K.

The 9-kb *Bgl*II fragment of pGKV562K carrying the *prtM*, *prtP*, and Km^r genes was then inserted into the unique *Bam*HI restriction site of pKL401B, thereby creating pKLG600. The integration vector pKLG610, with a functional *prtP* gene, was obtained by removing the 1.4-kb *Bam*HI fragment carrying the Km^r gene from the *prtP* coding sequence in pKLG600 (Fig. 1). All constructions were made with *B. subtilis* as a host.

Transformation. Protoplasts of *B. subtilis* were prepared and transformed as described by Chang and Cohen (3). *L. lactis* was transformed by electroporation as previously described (27).

Southern hybridizations. After electrophoresis in 0.8% agarose gels, the DNA (3 μg of chromosomal DNA per lane in each case) was transferred to GeneScreen Plus filters (Du Pont Co., NEN Research Products, Boston, Mass.) by the protocol of Southern, as modified by Chomczynski and Qasba (4). Probe labeling, hybridization conditions, and washing steps were according to the manufacturer's instructions for the ECL gene detection system (Amersham International, Amersham, United Kingdom). Labeled phage SPP1 DNA was added to the hybridization mixtures to facilitate easy determination of the sizes of the hybridizing fragments in the chromosomal digests.

Amplifications. The number of plasmid copies tandemly integrated in the *L. lactis* chromosome was determined with a Pharmacia LKB2222-020 UltraScan XL laser densitometer (LKB Produkter AB, Pharmacia, Bromma, Sweden).

Determination of stability. *L. lactis* MG1363(pGKV552), MG610, and MG611 were grown overnight in GM17. Then 0.1-ml samples of 10^{-3} dilutions of the cultures were inoculated in 100 ml of skim milk without antibiotics. After growth overnight (20 generations), the cultures were again diluted and 0.1-ml samples of 10^{-3} dilutions were inoculated in 100 ml of skim milk without antibiotics. This procedure was repeated until the cultures had been grown for 100 generations in the absence of antibiotics. After every 20 generations, diluted samples of the cultures were plated onto GM17 agar plates without antibiotics. The Em^r and Prt^+ phenotypes were tested by transferring 100 colonies of each strain from the nonselective plates to GM17 plates containing erythromycin and to GMAB plates.

Growth of mixed cultures. *L. lactis* MG1363, MG1363 (pGKV552), MG610, and MG611 were grown overnight in GM17, and then 10^{-3} dilutions were made. Each of the diluted Prt^+ Em^r cultures of strain MG1363(pGKV552), MG610, or MG611 was mixed with an equal volume (0.1 ml) of the 10^{-3} dilution of the Prt^- culture (MG1363) and inoculated in 100 ml of skim milk without antibiotics. The mixed cultures were grown for 60 generations in reconsti-

tuted skim milk without selective pressure at 30°C. Subculturing was as described above. After every 20 generations, dilutions of the mixed cultures were plated onto GM17 agar plates without erythromycin to determine the total colony count. The number of Em^r CFU was determined by replica plating at least 250 colonies to plates of GM17 agar containing erythromycin. The percentage of Em^r CFU represented the percentage of cells in the mixed culture still containing a proteinase plasmid, either replicating as in strain MG1363 (pGKV552) or integrated as in strains MG610 and MG611.

Proteinase activity assay. *L. lactis* MG1363, MG1363 (pGKV552), MG610, and MG611 were inoculated in whey permeate. After overnight growth, 50-μl samples of the cultures were added to a mixture of 150 μl of whey permeate, 50 μl of methoxysuccinylarginylprolyltyrosyl-*p*-nitroanilide (Kabi Diagnostica, Stockholm, Sweden), and 25 μl of $NaHPO_4$ (8). The mixtures were incubated for 15 min at 30°C, and then 50 μl of 80% (vol/vol) acetic acid was added to stop the reaction. After removal of the cells by centrifugation (3 min, 6,300 × *g*), 250 μl of the supernatant was transferred to a microtiter plate. The optical density at 405 nm (OD_{405}) was determined using a Titertek Multiskan MCC/340P spectrophotometer (Flow Laboratories Ltd., Rickmansworth, United Kingdom). The proteinase activity was determined by calculating the ΔA_{405} min^{-1} ml^{-1} of cultures adjusted to an OD_{660} of 1.

Proteinase isolation. *L. lactis* MG1363, MG1363(pGKV 552), MG610, and MG611 were grown to similar OD_{660}s during overnight growth in 20 ml of whey permeate. The cultures were centrifuged, and the pellets were washed once with 5 ml of 50 mM NaAc/P_i buffer (50 mM sodium acetate [pH 8] to which 50 mM NaH_2PO_4 was added until pH 6.5 was reached). After centrifugation the pellets were suspended in 0.5 ml of NaAc/P_i buffer. The suspensions were gently shaken at 30°C for 30 min and then centrifuged. The supernatants were stored at 0°C, and the pellets were resuspended in 0.5 ml of NaAc/P_i buffer. After 30 min of shaking at 30°C, the suspensions were centrifuged and the supernatants were combined with the first release fraction. The solutions were freeze dried in a SpeedVac Concentrator (Savant Instruments, Inc., Farmingdale, Conn.). The dried samples were resuspended in 100 μl of sterile water. Samples of the proteinase solutions were subjected to sodium dodecyl sulfate–7.5% polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (23). After electrophoresis, the gels were stained with Coomassie brilliant blue.

Determination of growth rate and acid production. Overnight cultures of *L. lactis* MG1363, MG1363(pGKV552), MG610, and MG611 grown in GM17 were diluted to a cell density of approximately 5 × 10^7 CFU/ml in 250 ml of reconstituted skim milk without antibiotics. The strains were grown for 10 h at 30°C; at 1-h intervals, samples were taken to determine the CFU per milliliter by plating onto GM17 agar plates without erythromycin and for the determination of acid production by titration with 0.1 NaOH.

RESULTS

Chromosomal integration of *prtM* and *prtP* by a Campbell-like mechanism. In previous work it was shown that the pTB19-based vector pKL400B can be stably integrated by a Campbell-like mechanism into the chromosome of the plasmid-free strain *L. lactis* MG1363 (27). This prompted us to examine whether pKL400B could be used as a vehicle to insert the *L. lactis* Wg2 proteinase genes *prtM* and *prtP* into the chromosome. For this purpose the 13.5-kb plasmid pKL610 was constructed (Fig. 1). *L. lactis* MG1363 was transformed by electroporation with 5 μg of pKLG610; two Em^r transformants were obtained and designated MG610 and MG611 (the transformation frequency with pGK12, a replicating lactococcal plasmid [20], was 4.5 × 10^5 transformants per μg in the same experiment). The Prt^+ phenotype of the two transformant strains was confirmed on GMAB agar plates. On this medium, strain MG1363(pGKV552) gave large yellow colonies with a yellow halo, whereas MG1363 produced small white colonies without a halo. Strain MG611 produced colonies with halos which were similar in color and size to those of strain MG1363(pGKV552). The colonies of strain MG610 were similar in size to those of strains MG611 and MG1363(pGKV552) but stained less intensively yellow, and the halos were smaller (results not shown).

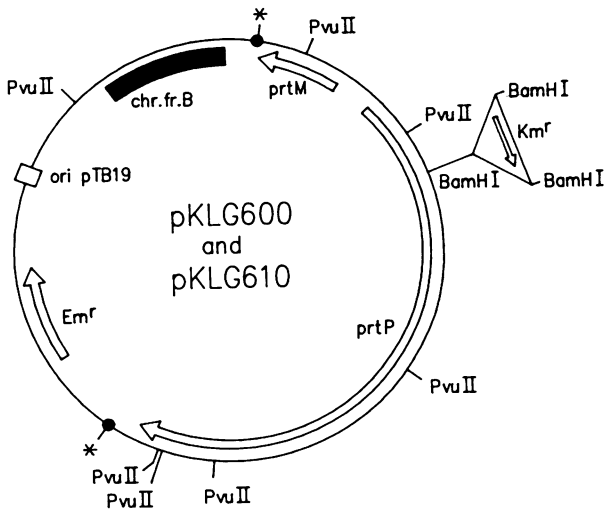

FIG. 1. Schematic representation of the integration plasmids pKLG600 (with the Km^r gene) and pKLG610 (without the Km^r gene). The solid box indicates the position of chromosomal fragment B. The asterisks indicate *Bam*HI-*Bgl*II fusion sites.

Southern hybridization analysis of *Pvu*II-digested chromosomal DNA of strains MG610 and MG611 with chromosomal fragment B as a probe demonstrated chromosomal integration of pKLG610 in both transformants. In *Pvu*II-digested chromosomal DNA of the recipient strain MG1363, as expected, only one hybridizing fragment at 3.6 kb was present (Fig. 2A, lane 6). Lanes 1 and 2 of Fig. 2A contained *Pvu*II digests of the chromosomal DNA of the transformant strains MG610 and MG611, respectively. Three fragments were present: one each at 3.3, 2.7, and 2.4 kb. The 2.4-kb fragment had the same size as the fragment that hybridized in the *Pvu*II digest of pKLG610 (lane 8). The patterns in Fig. 2A, lanes 1 and 2, indicate that pKLG610 had integrated in a Campbell-like manner into chromosomal fragment B and that a tandem arrangement of multiple copies of this plasmid was present on the chromosomes of the two transformant strains (Fig. 2B). The intensity of the 2.4-kb fragment as compared with those the 3.3- and 2.7-kb fragments in Fig. 2A, lanes 1 and 2, provides a measure of the extent of amplification. Although the numbers of integrated copies of pKLG610 were difficult to establish by visual inspection of Fig. 2A, densitometric determinations showed that the numbers of integrated copies of pKLG610 were two to three for strain MG610 and eight to nine for strain MG611.

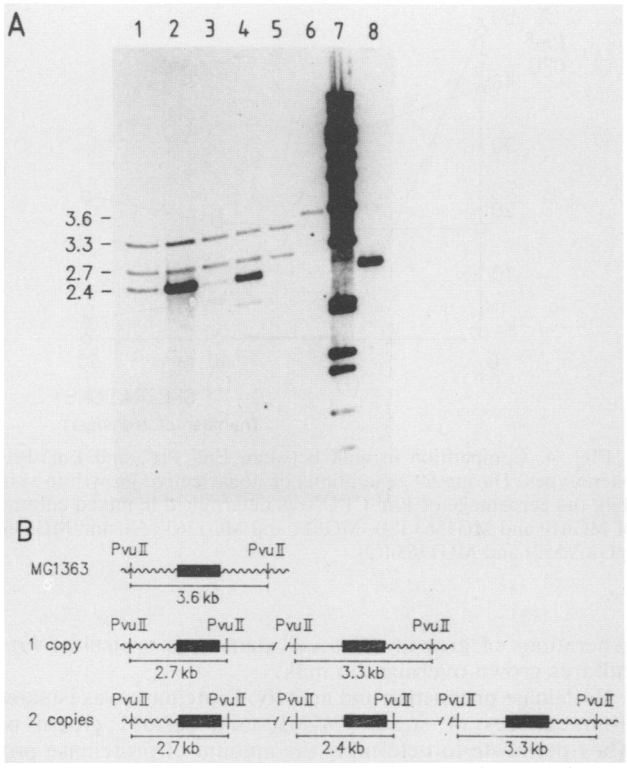

FIG. 2. (A) Southern hybridization analysis of *Pvu*II-digested chromosomal DNAs of strains MG610 (lane 1), MG611 (lane 2), MG610a (lane 3), MG610b (lane 4), MG610c (lane 5), and MG1363 (lane 6). Other lanes: 7, phage SPP1 DNA cleaved with *Eco*RI; 8, pKLG610 cleaved with *Pvu*II. Labeled chromosomal fragment B was used as a probe. Sizes (in kilobases) are indicated on the left. (B) Schematic representation of the relevant part of the chromosome of the host strain MG1363 and the expected structures after Campbell-like integration of pKLG610 with subsequent amplification. The solid boxes indicate the locations of chromosomal fragment B.

The number of integrated plasmid copies in strains MG610 and MG611 is slightly variable. In a second independently obtained chromosomal isolate of strain MG610, the intensity of the hybridization signal of the 2.4-kb fragment appeared to have decreased relative to that of the 3.3- and 2.7-kb fragments (results not shown). This observation prompted us to study strain MG610 in more detail. A culture of strain MG610 was plated onto GM17 plates, and five colonies, designated MG610a through MG610e, were randomly chosen for further analysis. Southern hybridization analysis of *Pvu*II-digested chromosomal DNA of the strains MG610a through MG610e with chromosomal fragment B used as a probe showed that cultures of strain MG610 were of a mixed nature. Chromosomal digests of the strain MG610a, MG610b, and MG610c are presented in Fig. 2A, lanes 3, 4, and 5, respectively. The levels of amplification in all five strains were determined densitometrically (Table 2). The number of integrated plasmid copies varied between one and six, with an average of approximately two, which was in agreement with the findings in the original isolate. The same procedure was used to analyze strain MG611. Four randomly chosen colonies, designated MG611a through MG611d, were examined (Table 2). The number of integrated plasmid copies varied between 6 and 10, indicating that cultures of strain MG611 also consisted of a mixed population.

TABLE 2. Plasmid amplification in transformant strains MG610 and MG611[a]

Strain	No. of tandemly arranged plasmid copies
MG610	2–3
MG610a	1–2
MG610b	5–6
MG610c	1
MG610d	1–2
MG610e	1
MG610-Ia	2–3
MG610-Ib	3–4
MG611	8–9
MG611a	7–8
MG611b	9–10
MG611c	6–7
MG611d	7–8
MG611-Ia	7–8
MG611-Ib	8–9

[a] The levels of amplification were determined densitometrically.

Stability of the Em^r and Prt$^+$ phenotypes of strains MG610 and MG611. The observed mixed nature of cultures of the strains MG610 and MG611 might result from instability of the integrated plasmids. To test the stability of the integrated structures, strains MG610 and MG611 were grown for 100 generations under nonselective conditions in skim milk. After every 20 generations, the Em^r and Prt$^+$ phenotypes of the strains were tested. Strain MG1363(pGKV552) was included in the assay to make a comparison with Em^r and proteinase genes carried by a replicative plasmid. No loss of the Em^r and Prt$^+$ phenotypes was observed in strains MG610 and MG611, whereas strain MG1363(pGKV552) was clearly unstable for both traits (Fig. 3). The somewhat higher rate of loss of the Prt$^+$ phenotype as compared with that of the Em^r phenotype of strain MG1363(pGKV552) during

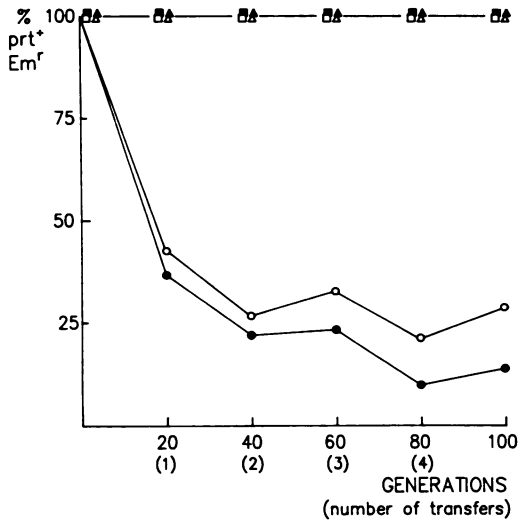

FIG. 3. Stability of the Prt$^+$ (■, ▲, ●) and Em^r (□, △, ○) phenotypes of strains MG610 (■, □), MG611 (▲, △), and MG1363(pGKV552) (●, ○) during nonselective growth in skim milk for 100 generations.

nonselective growth suggests that, in addition to plasmid loss, the genetic information for proteinase production was also subject to structural instability. The onset of coagulation of the milk caused by acid production was the same after every transfer during the 100 generations of growth for the strains MG610 and MG611. Strain MG1363(pGKV552) showed delayed coagulation after overnight growth of the second transfer, corresponding to growth for 60 generations.

The chromosomal structures of the cultures of strains MG610 and MG611 grown under nonselective conditions for 100 generations were examined. Two colonies of each strain were randomly chosen from nonselective plates and designated MG610-Ia, MG610-Ib, MG611-Ia, and MG611-Ib. Southern hybridizations revealed that the average level of amplification present in the strains MG610 and MG611 was maintained throughout the stability test (Table 2). Thus, the average number of integrated plasmid copies under nonselective growth conditions remained constant.

Growth of strains MG610 and MG611 cocultured with a Prt$^-$ strain. Although the average level of amplification was maintained during nonselective growth of the strains MG610 and MG611, the possibility cannot be excluded that occasionally cells may be produced that have lost all plasmid copies, resulting in Prt$^-$ cells that might overgrow the Prt$^+$ cells. It is well known that Prt$^+$ cells carrying the proteinase genes on an autonomously replicating plasmid are readily overgrown by Prt$^-$ cells that have lost the proteinase plasmid (34). To investigate whether Prt$^-$ cells that do not contain plasmids might overgrow cells containing several copies of the proteinase genes integrated in the chromosome, cultures of the Prt$^+$ strains MG610 and MG611 were mixed with a culture of the Prt$^-$ strain MG1363. The Prt$^+$ strain MG1363(pGKV552) mixed with the Prt$^-$ strain MG1363 was included as a control. The mixed cultures were grown under nonselective conditions in skim milk for 60 generations. After every 20 generations, dilutions of the cultures were plated onto GM17 plates without erythromycin to determine the total cell count. To determine the numbers of cells of strains MG610, MG611, and MG1363 (pGKV552) in the various mixed cultures, the colonies from the GM17 plates were replica plated onto GM17 plates with erythromycin. Strain MG1363 had no growth advantage over strain MG610 (carrying approximately two integrated plasmid copies) in the mixed culture during 60 generations, because the percentage of Em^r cells remained constant (Fig. 4). In contrast, the Prt$^-$ cells of strain MG1363 had a distinct growth advantage over the Prt$^+$ cells of strain MG611 (carrying approximately eight integrated plasmid copies). As expected, the cells of strain MG1363(pGKV552) were readily overgrown by the cells of strain MG1363. Notwithstanding the growth advantage of cells of strain MG1363 over those of strain MG611, the mixed culture with strain MG611 coagulated the milk as efficiently as did the mixed culture with strain MG610 throughout the experiment. The mixed culture with strain MG1363(pGKV552) showed delayed coagulation of the milk after overnight growth of the first transfer, corresponding to growth for 40 generations. These observations were in agreement with the total cell counts in the mixed cultures after overnight growth of the last transfer of the MG1363-MG610 and MG1363-MG611 mixtures, corresponding to 60 generations of growth, which both ranged from 2×10^9 to 4×10^9 CFU/ml. Such cell densities are indicative of good growth and rapid production of acid. In contrast, the mixed culture of MG1363 and MG1363(pKGV552) contained fewer than 7×10^8 CFU after overnight growth of the first transfer, corresponding to 40 generations of growth. This cell density is typical of Prt$^-$ cultures grown overnight in milk.

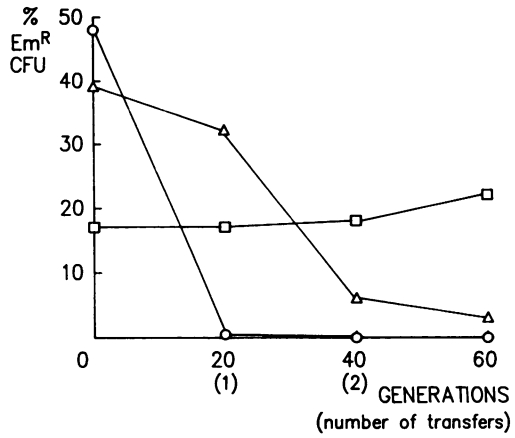

FIG. 4. Competition in milk between Em^r Prt$^+$ and Em^s Prt$^-$ phenotypes. During 60 generations of nonselective growth in skim milk the percentage of Em^r CFU was determined in mixed cultures of MG610 and MG1363 (□), MG611 and MG1363 (△), and MG1363 (pGKV552) and MG1363 (○).

Proteinase production and activity. Proteinase was isolated from cultures of strains MG610 and MG611 grown on whey-permeate to determine the amount of proteinase produced by these strains. Cultures of the strains MG1363 and MG1363(pGKV552) were used as controls. The samples were subjected to SDS-PAGE (Fig. 5). Strain MG611 produced slightly more proteinase than strain MG1363 (pGKV552) (lanes 3 and 4). The amount of proteinase produced by MG610 was minimal and just visible in the original gel (lane 2). The proteinase activities of strains MG610, MG611, MG1363(pGKV552), and MG1363 were determined by measuring the hydrolysis of the synthetic substrate methoxysuccinylarginylprolyltyrosyl-*p*-nitroanilide

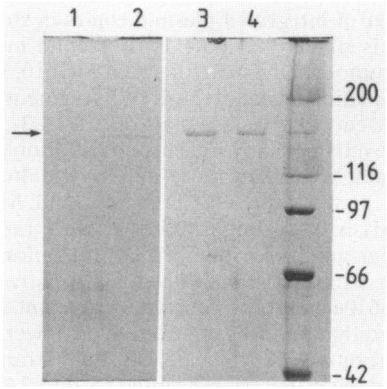

FIG. 5. Coomassie brilliant blue-stained SDS-PAGE gel of proteinase isolates of *L. lactis* strains. Lanes: 1, strain MG1363; 2, strain MG610; 3, strain MG611; 4, strain MG1363(pGKV552). In lanes 1 and 2 the equivalents of 100 μl of the cultures were used; in lanes 3 and 4 the equivalents of 25 μl of the cultures were used. High-molecular-weight standards are shown in the right-hand lane: myosin (200,000), β-galactosidase (116,250), phosphorylase *b* (97,400), bovine serum albumin (66,200), and ovalbumin (42,699). Molecular sizes (in kilodaltons) are shown on the right. The arrow indicates the position of the proteinase.

TABLE 3. Proteolytic activity of *L. lactis* strains[a]

Strain	ΔOD_{405}[b]/min/ml
MG1363	0
MG610	0.053
MG611	0.619
MG1363(pGKV552)	0.415

[a] The proteolytic activities of cultures adjusted to an OD_{660} of 1 were determined by using the synthetic substrate methoxysuccinylarginylprolyltyrosyl-*p*-nitroanilide.
[b] Change in OD_{405}.

(Table 3). The proteolytic activity of strain MG611 was approximately 1.5 times higher than that of strain MG1363 (pGKV552) and approximately 11 times higher than that of strain MG610. Quantitatively, these results are in agreement with the amounts of proteinase that were obtained from these strains (Fig. 5).

Growth rate and acid production. Strains MG610 and MG611 differed considerably in the number of integrated proteinase genes and consequently in the amount of active proteinase produced. Furthermore, strain MG611 produced more active proteinase than did MG1363(pGKV552), which carried the same proteinase gene on an autonomously replicating plasmid. The effect of the differences in proteolytic activity of these strains on the growth rate and acid production in milk was determined. The cultures were inoculated to a cell density of approximately 5×10^7 CFU/ml, and growth was allowed for 10 h in the absence of antibiotics. The number of cells in the cultures and the amount of acid produced were determined at intervals of 1 h (Fig. 6). The Prt$^-$ strain MG1363 stopped growing at a cell density of approximately 7×10^8 CFU/ml as a result of the depletion of the available free amino acids in the milk and produced only small amounts of acid (up to 25 μmol/ml). Strains MG610, MG611, and MG1363(pGKV552) were able to grow to higher cell densities (approximately 2×10^9 CFU/ml) and produced considerably more acid (45 to 60 μmol/ml). The initial growth rate and acid production of strain MG610 (containing approximately two integrated plasmid copies) were higher than the initial growth rates and acid production rates of the strains MG611 (containing approximately eight integrated plasmid copies) and MG1363(pGKV552) (approximately five plasmid copies [20]), which were mutually similar. However, after the depletion of the free amino acid pool (after 3 h, when the Prt$^-$ strain MG1363 stopped growing), the growth rate of strain MG610 became less than those of strains MG611 and MG1363(pGKV552). As a result, the stationary phase (at a similar cell density) was reached approximately at the same time (after 7 h) by the three strains. After 10 h of incubation, strain MG611 had produced the highest amount of acid in all cases. These results suggest that only a few copies of the proteinase genes are sufficient for good growth and rapid production of acid in milk.

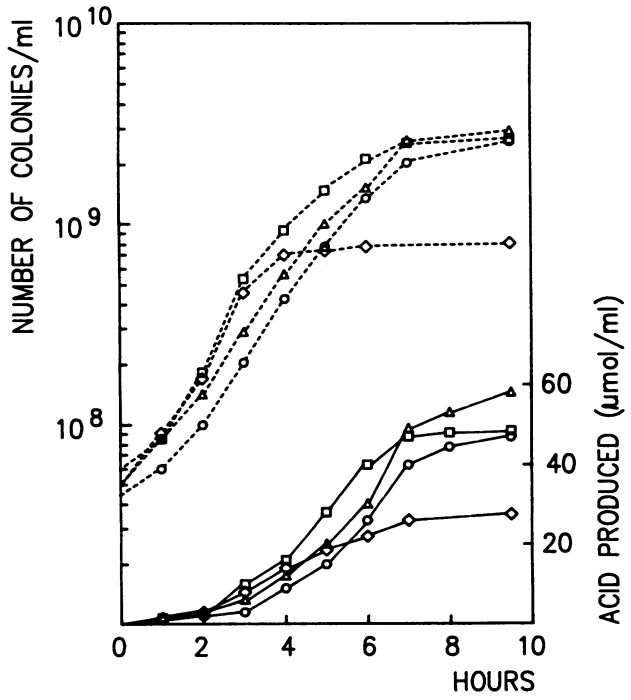

FIG. 6. Growth (– – –) and acid production (——) in skim milk of the strains MG610 (□), MG611 (△), MG1363(pGKV552) (○), and MG1363 (◇) as a function of time.

DISCUSSION

Using the pTB19-derived insertion vector pKLG610, we have demonstrated that the unstable plasmid-encoded lactococcal proteinase genes can be stabilized by Campbell-like integration into the *L. lactis* chromosome. The transformant strains MG610 and MG611 contained different numbers of pKLG610 in tandem arrangement on their chromosomes. The amplified structures were obtained without the need to grow the transformant strains on elevated levels of antibiotics, and the amplifications were stably maintained during nonselective growth. We have previously reported the integration of the pTB19-derived integration vector pKL400B, from which pKLG610 was derived. The transformants obtained with pKL400B contained only a single integrated plasmid copy, and amplification was not observed (27). Since the major difference between pKL400B and pKLG610 concerns the presence of the *prtP* and *prtM* genes in pKLG610, it is tempting to speculate that the difference in plasmid structure underlies their different behavior with respect to amplification.

The amplifications in the transformants described here differ in one respect from amplifications obtained by others in *L. lactis* (5) and other bacteria (10, 13, 36). Amplification of plasmids integrated via a Campbell-type mechanism was observed by others after the bacteria were challenged with increasing concentrations of antibiotics. Chopin et al. (5) used the same selectable marker (Em^r) as the one used here to integrate pE194 into the *L. lactis* IL1403 chromosome. Amplification was only observed after growth of the transformants on increasing concentrations of clindamycin. Although other possibilities cannot be excluded, these differences may be related to the origin of the homologous segment of DNA employed in the insertion vector to effect integration. Immediate amplification of integrated plasmids in *B. subtilis* after selection of the transformants on a low concentration of antibiotic was also reported by Young (42, 43). However, these amplifications proved to be unstable, and recently it was shown that residual replicative activity of the integrated replicon was involved in the instability (44).

In previous work we have suggested that stable spontaneously amplified plasmids in the chromosome of *L. lactis* might result from the integration of plasmid multimers (27). Strain MG610 may be the result of the integration of a plasmid multimer, but it is unlikely that strain MG611 is the

product of such an event because this strain carried on the average eight integrated plasmid copies. The high stability of the different levels of amplification in strains MG610 and MG611 suggests that this level is determined shortly after the integration of the first plasmid copy. One possibility to explain the difference in the presence of only few tandemly repeated integrated plasmid molecules (as in strain MG610) and the presence of many (as in strain MG611) is based on the admittedly speculative assumption that differential damage inflicted upon the cells during the electrotransformation procedure allows for differences in the number of plasmid molecules that enter into the cells. If one of the internalized plasmid copies becomes integrated in the chromosome, subsequent integration of additional internalized copies by homologous recombination may result in multiple integrated copies, the number of copies ultimately being dependent on the number of plasmids internalized. Although the average level of amplification is apparently stable, as shown in the subculturing experiments, individual clones show variation. Apparently, an additional plasmid copy can be lost or gained, possibly by unequal crossing over between daughter chromosomes or by intrastrand recombination among the tandem repeats.

The proteinase activity of the transformant strains depended on the level of amplification of the proteinase genes. Such a gene dosage effect has also been reported for other bacteria in which genes were amplified after a Campbell-like integration (1, 10, 13, 14, 32, 36). The difference in proteolytic activity of the strains MG610, MG611, and MG1363 (pGKV552) did not influence the growth characteristics and acid production in milk in a major way, although strains MG611 and MG1363(pGKV552) showed a higher growth rate after the depletion of the free amino acid pool and strain MG611 consistently produced more acid. The initial growth rate of strain MG610 was the same as that of the Prt^- strain MG1363 for as long as the free amino acid pool was not depleted. This result is in agreement with the observation that in mixed cultures of these two strains there is no growth advantage of strain MG1363 over strain MG610 and suggests that a limited number of integrated copies of the proteinase genes per genome does not have an adverse effect on the initial growth rate. In contrast, the initial growth rates of strains MG611 and MG1363(pGKV552) were less than those of strains MG1363 and MG610, most likely because of the large number of proteinase genes in the chromosome of strain MG611, the presence of an autonomously replicating proteinase plasmid in strain MG1363(pGKV552), or the presence of large amounts of proteinase in both of these strains. These results are in agreement with the observed decrease in CFU of strains MG611 and MG1363(pGKV552) in the mixed cultures with strain MG1363, although the decrease of strain MG611 was less pronounced (Fig. 4). The fact that a strain like MG610, with reduced proteinase activity, showed good growth and rapid production of acid in milk is in agreement with earlier observations. McKay and Baldwin (31) observed integration into the chromosome of a proteinase plasmid after transduction experiments with *L. lactis* C2. Although the proteinase activity of the transductant was 50% less than that of the parental strain, the transductant was successfully used to make Cheddar cheese, which was less bitter than cheese produced with the original strain (15). The overproduction of proteinase in strain MG611 might have a favorable effect on cheese ripening time. It is known that addition of commercially available food grade proteinases can accelerate cheese ripening (25).

In summary, we conclude that the Campbell-like integration strategy can be used to stabilize lactococcal strains for genes that are important in the dairy industry and that such genes may be subject to substantial amplification.

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